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Increased Drug-Metabolizing Activity without Increased Cytochrome P-450 in Rats Treated with Phenolic Antioxidants

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The endoplasmic reticulum of the liver contains a cytochrome (*P*-450) that has been implicated in the enzymic oxidation of drugs and other foreign compounds (Cooper, Levine, Narasimhulu, Rosenthal & Estabrook, 1965). Since the activities of several of these oxidative enzymes are increased in rats that have been treated with various phenolic compounds (Gilbert, Martin & Golberg, 1967), the cytochrome *P*-450 content of the livers of similarly treated rats was measured. Most of the compounds that increased the enzyme activities also increased the cytochrome *P*-450 content of the liver, but two compounds caused several-fold increases in the drug-metabolizing enzymes with little or no change in cytochrome *P*-450 content. These two compounds were 2,6-di-*tert*.-butyl-4-methylphenol (BHT; I) and 2,6-di-*tert*.-butyl-4-methoxymethylphenol (II).

The livers of female rats (100g.) were removed 24 hr. after an oral dose (1.5 m-moles/kg.) of these test compounds. Activities of the microsomal enzyme BHT oxidase (Gilbert & Golberg, 1965) were (expressed as means $\pm$ s.e.m., with numbers of values in parentheses): controls, 0.26 ± 0.04 (4), compound I, 0.78 ± 0.07 (3), and compound II, 1.15 ± 0.02 (4) μ moles/hr./g. of liver. The cytochrome *P*-450 contents of the same livers were: controls, 9.2 ± 0.7 , compound I, 10.5 ± 0.8 , and compound II, 12.4 ± 0.7 nmoles/g. of liver.

A similar result was obtained by using the duration of action of hexobarbitone to measure the rate of drug metabolism *in vivo*. At 48 hr. after doses of 0.2, 0.5 or 1.5 m-moles of compound II/kg. the hexobarbitone sleeping times of female rats (100g.) were significantly decreased (75, 65 and 48% of control respectively; groups of four to six), indicating accelerated hexobarbitone oxidation. The cytochrome *P*-450 contents of the livers of identically treated rats were not significantly changed (107, 121 and 107% of control respectively; groups of six). At 48 hr. after a dose of phenobarbitone (100 mg./kg.) both the hexobarbitone

sleeping time and the cytochrome *P*-450 content were significantly altered (27% and 232% of control respectively; groups of six).

It appears from these experiments that cytochrome *P*-450 may not be rate-limiting for oxidative drug metabolism *in vivo* or *in vitro*.

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The Haem-Protein Interaction in Cytochrome P-450

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In order to understand the many spectroscopic peculiarities associated with cytochrome *P*-450 we have made a series of model studies on protohaem complexes, especially those in which the iron is further bound to a thiol. The ferrihaem complexes with thiols give rise to absorption and electron-proton-resonance spectra that closely resemble those of cytochrome *P*-450 and are compatible with the ferric iron being largely in a low-spin state (Bayer, Hill, Roder & Williams, 1969). However, there is always some high-spin ferric iron present and the amount of this form of the iron is dependent on the substrate added (Williams, 1968). As these substrates can not bind the iron in general it follows that they must modify the iron-protein bonding.

The spectroscopic properties of ferrocytochrome *P*-450 indicate that the iron is high-spin. Such ferrohaem complexes have very little affinity for thiols. We conclude that the curious difference spectra of cytochrome *P*-450, oxidized minus reduced, are due to the unusual spin states of the iron, ferric iron low-spin and ferrous iron high-spin, and to the weakening or breaking of the iron-sulphur bond on reduction. On addition of carbon monoxide, nitric oxide or isonitriles ferrocytochrome *P*-450 has absorption bands at 450 and 550 nm. but with no α -band. Model studies show that this type of spectrum is most closely approached when metal atoms in porphyrin complexes become five-co-ordinate and the metal lies out of the plane of the porphyrin by some considerable distance. This state of the metal, should it also arise on reaction with oxygen, would lead to a very open complex that is similar to the five-co-ordinate forms of vitamin B₁₂ derivatives. Vallee & Williams (1968) have referred to such unusual co-ordination conditions as entatic states.

Cytochrome *P*-450 is a two-electron oxidizing agent when combined with oxygen and linked to NADH, although the overall reaction involves four electrons, two from the NADH. The steps of

the reaction could well involve $\left[\text{RS}^- \text{Fe(II)O}_2\text{-XH} \right]$

where XH is the substrate, $\left[\text{RS}^\cdot \text{Fe(III)O}_2^{2-}\text{-XH} \right]$,

and $\left[\text{RS}^\cdot \text{Fe(III) + XOH + H}_2\text{O} \right]$, before the last com-

plex is reduced back to $\left[\text{RS}^- \text{Fe(II)} \right]$. The very low redox potential of cytochrome *P*-450 is consistent with an anionic group such as a thiol as the proximal group to the haem.

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The Metabolism of some Substituted Benzofuran β -Adrenergic Blocking Agents

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From a number of substituted benzofurans with β -adrenoceptive blocking properties, 2-(1-hydroxy-2-isopropylaminoethyl)-6,7-dimethylbenzofuran (Ro 3-3528) was selected for clinical trial, and its metabolism studied in the rat, mouse, dog and man.

The principal metabolite in all species was 6-carboxy-2-(1-hydroxy-2-isopropylaminoethyl)-7-methylbenzofuran isolated as its methyl ester and identified by n.m.r. and mass-spectral measurements. In man this compound accounted for 90% of the urinary radioactivity. From urine of rats treated with Ro 3-3528, 6,7-dimethylbenzofuran-2-ylglycollic acid and the corresponding glyoxylic acid and carboxylic acid were identified on the basis of mass-spectral determination, chemical properties and reverse isotope dilution with authentic substances. These metabolites accounted for 5% of the urinary radioactivity in man, 15% in the rat and 25% in the dog. A small amount of the primary amine formed by *N*-dealkylation of Ro 3-3528 was found in rat urine. This compound was found to be a good substrate for mitochondrial monoamine oxidase. Since treatment of rats with the monoamine oxidase inhibitor Pargyline had only a slight effect on the quantity of primary amine excreted after Ro 3-3528 had been given, oxidative deamination of this metabolite is catalysed by other enzymes.

The other identified metabolite from rat urine was a phenol derived by hydroxylation of Ro 3-3528 in the 5- or possibly the 4-position. Identification was based on mass-spectral, n.m.r. and u.v. measurements and on changes in the acid-lability of ^3H label originally present in the 5-position of the benzofuran ring. This compound made up 25% of the metabolites in the urine in the rat, 5% in the dog and was not detected in human urine.

In man 90% of administered radioactivity appeared in urine in 48 hr. In the rat the 6-carboxy metabolite was excreted as rapidly in bile as in urine, despite its relatively low molecular weight (278).

Distribution of the drug was studied in the mouse by whole-body radioautography. The duration of β -blocking activity correlated with the decline of tissue drug concentrations as measured fluorimetrically. These concentrations were much higher in female than male rats and in male than female mice, in accord with known sex-determined differences in drug-metabolizing enzymes in these species.

Analogous benzofurans lacking a 6-substituent were metabolized in the rat by 6-hydroxylation. For example, the metabolites of 2-(1-hydroxy-2-isopropylaminoethyl)benzofuran in the urine were the glucuronide of the 6-hydroxy compound (75%) and a mixture of acids obtained by oxidative deamination (25%).

Quinate Metabolism by *Lactobacilli*

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Lactobacillus pastorianus, a typical heterofermentative cider lactobacillus, reduces (–)-quinic acid and shikimate to dihydroshikimate (Carr, Pollard, Whiting & Williams, 1957). The enzyme system responsible has been found to be adaptive. Adapted washed cells incubated anaerobically with quinate or shikimate, in the absence of a hydrogen donor, formed an equimolecular mixture of the two. In the presence of quinate or shikimate, the rate of CO_2 formation from fructose by adapted cells was increased sevenfold. Of the usual products of fructose metabolism, namely mannitol, lactate, acetate and CO_2 , neither mannitol nor lactate was found, only acetate and CO_2 , together with dihydroshikimate. In the absence of shikimate washed cells metabolized glucose at only one-twentieth the rate of fructose, but in its presence the rates of CO_2 formation from glucose and fructose were identical. Aerobically, washed cells in the absence of an artificial electron acceptor oxidized lactate